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High Performance Liquid Chromatography (HPLC)

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ABSTRACT

High Performance Liquid Chromatography (HPLC) is a process of separating components in a liquid mixture. A liquid sample is injected into a stream of solvent (mobile phase) flowing through a column packed with a separation medium (stationary phase). Sample components separate from one another by a process of differential migration as they flow through the column.

As bands emerge from the column, flow carries them to one or more detectors which deliver a voltage response as a function of time. This is called a chromatogram. For each peak, the time at which it emerges identifies the sample constituent with respect to a standard. The peak's area represents the quantity.

HPLC provides a highly specific, reasonably precise, and fairly rapid analytical method for a plethora of complicated samples.

This is difficult in detecting compounds. Low sensitivity of some compounds towards the stationary phase in the columns is difficult.

KEYWORDS: *High-performance liquid chromatography (HPLC), Types, Advantages, Disadvantages*

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INTRODUCTION

High-performance liquid chromatography (HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phases through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile). Sepration has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte. (1)

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DEFINITION

High performance liquid chromatography (HPLC) formerly refers to as high pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. it relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid absorbent material full touch. each component in the sample interacts slidely differently with the absorbent materail, causing diffenent flow rates for the different components and leading to the seperation of the componenets as they flow out of the column. [3]

HISTORY

Liquid chromatographic systems were to an inefficient because of the flow rate of solvents being reliant on gravity. Separations took numerous hours, and some of the time days to finish. Gas chromatography (GC) at the time was more effective than liquid chromatography (LC), in any case, it was trusted that gas stage partition and investigation of extremely polar high atomic weight biopolymers was impossible. GC was ineffectual for some organic chemists due to the thermal instability of the solutes. Accordingly, alternative techniques were hypothesized which would soon bring about the advancement of HPLC. Taking after on the original work of Martin and Synge in 1941, it was anticipated by Cal Giddings, Josef Huber, and others in the 1960s that LC could be worked in the high proficiency mode by decreasing the pressing molecule measurement generously beneath the run of the mill LC (and GC) level of 150 µm and utilizing pressure to expand the versatile stage velocity. These expectations experienced broad experimentation and refinement all through the 60s into the 70s. Early developmental exploration started to enhance LC particles, and the innovation of Zipax, an externally permeable molecule, was promising for HPLC technology. The 1970s achieved numerous advancements in equipment and instrumentation. Specialists started utilizing pumps and injectors to make a simple configuration of a HPLC system. Gas amplifier pumps were perfect since they worked at consistent pressure and did not require release free seals or check valves for steady flow and great quantitation. While instrumentational advancements were important, the historical backdrop of HPLC is principally about the history and development of molecule technology. After the presentation of permeable layer particles, there has been a steady pattern to reduced molecule size to enhance efficiency. However, by decreasing molecule size new issues arrived. The disadvantage from the unnecessary pressure drop is expected to drive versatile liquid through the segment and the trouble of setting up a uniform pressing of to a great degree fine materials. Every time molecule size is diminished altogether, another round of instrument advancement normally should occur to handle the pressure. [4]

DIFFERENT TYPES OF HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process:

1. Normal phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these.

Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

2. Reversed phase chromatography

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

3. Ion exchange chromatography

In Ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase.

Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, Ligand-exchange chromatography,

Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc.

4. Size exclusion chromatography

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids.

This technique is widely used for the molecular weight determination of polysaccharides 5. Bio-affinity chromatography.

Separation based on specific reversible interaction of proteins with ligand. Ligand are covalently attached to solid support on a bio-affinity matrix, retains

proteins with interaction to the column-bound ligand. Proteins bound to a bioaffinity column can be eluted in two ways:

Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand.

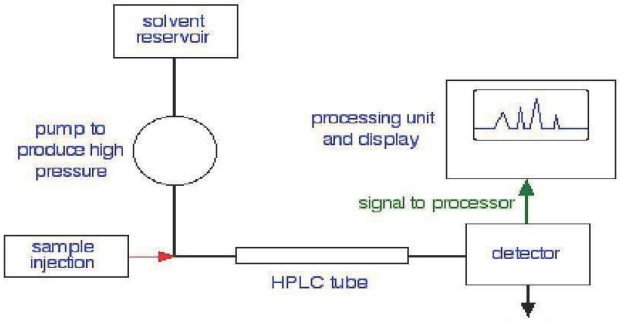
Aspecific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.

Because of specificity of the interaction, bioaffinity chromatography can result in very high purification in a single step (10 - 1000-fold) [5]

OPERATION

The sample to be analyzed is injected in a small volume into the stream of the mobile phase. The motion of analyte through the column is slowed by specific chemical or physical interactions with the stationary phases as it traverses the length of the column. The amount the analyte is slowed depends on the nature of the analyte and on the compositions of the stationary and mobile phases. Time taken by a specific analyte to elute is called retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. Smaller particle size column packing (which creates a higher back-pressure) increases the linear velocity giving the components less time to diffuse within the column, which leads to improved resolution in the resulting chromatogram. Commonly used solvents include any miscible combination of water or various organic liquids (most common being methanol and acetonitrile). Water may contain buffers or salts to help in separation of the analyte components or compounds such as trifluoroacetic acid which acts as an ion pairing agent. A further refinement to HPLC has been to change the mobile phase composition during the analysis. This is known as gradient elution. A general gradient for reversed phase chromatography might start at 5% methanol and progresses gradually to 50% methanol over 25 minutes; the gradient chosen depends on the hydrophobicity of the analyte. The analyte mixtures are separated as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This process of partioning is similar to that which occurs during a liquid-liquid extraction but this is continuous and not step-wise. For example, when using a low water/ high methanol gradient, the more hydrophobic components will elute from the column due to a relatively hydrophobic mobile phase. The hydrophilic compounds will elute under conditions of relatively low methanol/high water. The choice of solvents, additives and gradient depend on the nature of the analyte and the stationary phase. Generally a series of tests are performed on the analyte and a number of trial runs may be processed in order to find the optimum HPLC method giving the best separation of peaks. [6]

A flow scheme for HPLC



APPLICATIONS OF HPLC

The HPLC has several applications in the fields of pharmacy, forensic, environment and clinical. It also helps in the separation and purification of compound.

- Pharmaceutical Applications: The pharmaceutical applications include controlling of drug stability, dissolution studies and quality control.
- Environmental Applications: Monitoring of pollutants and detecting components of drinking water.
- Forensic Applications: Analysis of textile dyes, quantification of drugs and steroids in biological samples.
- Food and Flavour Applications: Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives.
- Clinical Applications: Detecting endogeneous neuropeptides, analysis of biological samples like blood and urine.[7]

ADVANTAGES

HPLC has many advantages over other methods of chromatography. It has made significant contribution to the growth of analytical science and its diverse application in pharmaceuticals, environmental, forensics, foods, polymers and plastics, clinical fields etc. HPLC provides a highly specific, reasonably precise, and fairly rapid analytical method for a plethora of complicated samples. HPLC is capable of tackling macromolecules It is profoundly suitable for most 'pharmaceutical drug substances'. It offers an efficient means of analysis pertaining to 'labile natural products'. HPLC allows the dependable analysis of a good number of products including biochemical, metabolic products, nonvolatile substance, polar compounds etc. Preparation and

introduction of sample is easy and simple in HPLC. Resolution of compounds and speed of separation is high. HPLC software is capable of reporting precise and accurate results. Sensitivity of detectors used is high. A large number of stationary phases and columns can be used to suit different ranges of application. Recording and storage of information is easy. The columns operated carefully under controlled conditions without overloading can be resed for significant period of time. HPLC coupled with mass spectrophotometers and FT- IR system have improved efficacy. Along with hyphenated techniques HPLC have been used to analyse impurities in pharmaceutical formulations. [8]

DISADVANTAGES

HPLC is considered one of the most important techniques of the last decade of the 20th century. Despite of the several advantages there are certain limitations also. Limitations include price of columns, solvents and a lack of long term reproducibility due to proprietary nature of column packing. Others include: Complexity of separation of certain antibodies specific to the protein. The cost of developing an HPLC apparatus for assay or method of separation of individual components is tremendous. Due to the speed of the HPLC and its reliance on the different polarities; two compounds with similar structure and polarities can exit the chromatographic apparatus at the same time (co-elution). This is difficult in detecting compounds. Low sensitivity of some compounds towards the stationary phase in the columns is difficult. Certain compounds get absorbed or react with the chemicals present in the packing materials of the column. Sometimes the pressure may get too high or low that the column cannot withstand or separation may not takes place. Qualitative analysis may be limited unless HPLC is interfaced with mass spectrometry. Resolution is limited with very complex samples. Newer trends with better efficacy have been established. [8]

CONCLUSION

High-performance liquid chromatography (HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phases through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. The sample to be analyzed is injected in a small volume into the stream of the mobile phase. The motion of analyte through the column is slowed by specific chemical or physical interactions with the stationary phases as it traverses the length of the column. The amount the analyte is slowed depends on the nature of the analyte and on the compositions of the stationary and mobile phase.

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